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# Precipitation of Detergent-Containing Samples for Top-Down and Bottom-Up Proteomics

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## Abstract

Prior to proteome analysis by mass spectrometry (MS), protein mixtures must first be subject to various sample preparation steps. The goal is to isolate proteins in high yield, and with high purity. Liquid chromatography (LC) separation is also integral to comprehensive proteome characterization, and so a key component of sample preparation is simply to solubilize the proteome in LC-MS compatible solvents. Hydrophobic proteins (membrane proteins) represent a greater challenge to maintain protein solubility during sample preparation. Sodium dodecyl sulfate (SDS) is a favored detergent to solubilize proteins, and also is used to impart mass-based fractionation (i.e., SDS PAGE, GELFrEE). However, SDS is incompatible with downstream LC-MS analysis. Fortunately, effective strategies for SDS removal do exist, which permits the use of this surfactant in proteomics workflows. Here we highlight an approach that is grounded in the classic technique—protein precipitation. The technique has been updated and has recently seen a revival as a strategy permitting high protein recovery, with exceptional purity. Moreover, with aid of simple disposable spin cartridges, protein precipitation can meet the needs of high throughput, automated, and reproducible proteome purification, enabling the analysis of SDS-containing samples in both top-down and bottom-up formats.

**Keywords:** sample preparation, mass spectrometry, sodium dodecyl sulfate, GELFrEE, acetone precipitation, ion pairing, protein modification, ProTrap XG

## 1. Introduction

In proteomics, mass spectrometry (MS) is the tool of choice for comprehensive analytical characterization. Both qualitative identification and quantitative profiling of individual proteins in the mixture are made possible through MS, noting that distinct detection workflows are available to characterize the system. Acknowledging the multiple advances in MS instrumentation as primary contributors to the tremendous growth in proteome characterization, it is essential to realize that MS represents but one component in a complex proteome workflow. Successful proteome characterization ultimately depends on the proper isolation and delivery of purely resolved proteins in high yield to the analytical detection platform. The establishment of consistent “front-end” approaches which can ensure the removal of MS interferences, with unbiased recovery of all protein components, in LC-MS compatible format is an essential first step of the detection platform.

## 1.1 Top-down versus bottom-up proteomics

Two distinct and complementary workflows exist to characterize proteome samples by mass spectrometry: the bottom-up proteomics (BUP) and top-down proteomics (TDP) methods [1]. BUP is considered the more classical and also most widely used approach to MS-based proteome characterization. In BUP, proteins are first hydrolyzed into smaller peptide segments, typically using an enzyme such as trypsin. Trypsin cleaves at specific amino acid residues, namely on the C-terminal side of lysine and arginine [2], giving rise to predictable peptides possessing a favorable mass range (~1–2 kDa) and charge state (+2 or higher), both of which encourage optimal MS detection. These smaller peptide chains are separated by liquid chromatography (LC), which can be directly coupled to MS by way of electrospray ionization (ESI). Small peptides are readily resolved through reversed phase liquid chromatography. If necessary, they can even be further fractionated by way of orthogonal modes of separation such as ion exchange chromatography [3]. As the peptides elute from the LC column and are directed to the gas phase by ESI, the resulting ions are first profiled by MS to determine the molecular weight of the precursor (intact) peptide molecule. These gas phase molecules are then fragmented, and through a second dimension of mass spectrometry (i.e., tandem MS, or MS/MS), the resulting spectra reveal the mass of the fragments. With aid of computer programs, the tandem MS spectra ultimately carry the mass information to decipher the amino acid sequence of the peptides, which are correlated back to the original protein. BUP offers the potential for deep proteome coverage; using state-of-the-art MS instrumentation, coupled with appropriate separation, it is essentially possible to detect “all” unique proteins of simple proteome systems [4, 5]. Further to this, quantitative analysis is readily possible, using internal [6] or external [7] calibration standards often generated through isotope labelling to correlate MS intensity data with sample concentration.

While BUP is a widespread approach, several researchers have recognized the limitations of the strategy to attain the goals of proteome profiling. In particular, while the detection of multiple peptides stemming from distinct proteins of a proteome mixture does demonstrate the presence of a protein, it is rarely possible to observe “all” peptides stemming from a given protein. In other words, protein sequence coverage is often incomplete. It is possible that important regions of a protein, for example, one containing a post translational modification, may go undetected. Post-translational modifications are but one of the factors leading to greater protein diversity that would be described by a simple translation of the genome into a proteome. As such, researchers have used the word “proteoform” to describe the distinct chemical entities generated from slight variations in proteins [8]. For example, consider hypothetical protein which might carry 10 possible post-translational modifications. Even if these modifications are binary in nature (i.e., present or not), this would lead to  $2^{10}$  (1024) distinctly modified forms (modforms) for this one protein [9]. Though this simple example may be an over-estimation of protein diversity, it is clear that the proteome carries with it a greater chemical diversity than the genome from which it is derived. Together with dynamic changes in expression, the functional diversity of an organism is only captured through characterization of distinct proteoforms. Given the importance of complete proteome characterization, MS detection workflows should be cognizant of providing data which captures this increased diversity in the sample. BUP methods are generally not suited to profile distinct proteoforms, since the digestion process will not retain the combination of protein modifications that would be retained at the intact molecular level.

By contrast to BUP, with top-down proteomics, proteins are detected by mass spectrometry without subjecting them to enzymatic digestion. Thus, the gas phase protein ions are generally of much higher molecular weight (~10–100 kDa), and

also of higher charge state ( $\sim +5$  to  $+50$ ). These higher masses demand higher resolution MS platforms, as well as fragmentation approaches suited to generate tandem MS spectra for the intact proteins [10]. Like BUP, the TDP approach also necessitates front-end separation, as well as computational tools to interpret the resulting spectra. Given that the TDP approach begins with the entire protein molecule, it is theoretically possible to localize all possible modifications present on the molecule [11]. In practice, localization of these modifications depends on the quality of the resulting fragmentation spectrum. TDP has grown steadily in its ability to detect an increasing number of proteins and proteoforms in a system. Nonetheless, TDP is still considered to be more technically challenging. Several of these challenges stem from issues related to front-end sample preparation. These include greater challenges related to maintaining protein solubility, both during and after proteome fractionation, together with issues surrounding protein purity which impacts MS sensitivity. In short, while TDP offers greater potential for proteoform characterization, proper sample preparation is even more critical to the success of the approach.

## 1.2 Protein solubility

The digestion of a protein into peptide fragments offers immediate advantages in terms of easier sample handling. Peptides are generally more soluble than their intact protein counterparts. Certainly, there is likely to be at least one portion of the digested protein that is more soluble and also more readily detected by MS (the proteotypic peptide) [12]. In sharp contrast, the chemical diversity existing at the level of intact proteins is far greater. Proteins vary more greatly in size, charge, and polarity [13]. They also adopt various folding states and therefore can behave unpredictably throughout the various stages of sample preparation.

The solubility of an intact protein is highly influenced by the tertiary structure of the protein. In aqueous systems, proteins adopt a folded structure which shields the more hydrophobic amino acids, while exposing polar or charged residues on their outside surface. This favors the formation of an ordered hydration layer in order to shield the electrostatic regions of the protein; therefore decreasing electrostatic protein-protein interactions. At low ionic strength the shielding of the protein by the hydration layer can be described by the Debye-Huckel theory, which assumes that the protein is surrounded by ions of opposite charge [14]. This causes a reduction in the electrostatic free energy of the protein, resulting in a decrease in activity. This solubility can be described by the equation:

$$\ln(s_2/s_{2,w}) = Z^2 \epsilon^2 N \kappa / [2DRT(1 + \kappa a)] \quad (1)$$

where  $Z$  is the overall net charge,  $\epsilon$  is the electronic charge,  $N$  is Avogadro's number,  $D$  is the dielectric constant,  $R$  is the universal gas constant,  $T$  is the temperature,  $a$  is the radius of the ionic cloud,  $I$  is the ionic strength and  $\kappa$  is given by:

$$\kappa = \sqrt{8\pi N \epsilon^2 / 1000 D k T} \sqrt{I} \quad (2)$$

$$I = \frac{1}{2} \sum_i C_i Z_i^2 \quad (3)$$

This description of protein solubility only accounts for the electrostatic forces of the protein and not the hydrophobic interactions. Protein solubility in aqueous systems has been further described by two mathematical models, the osmotic second virial coefficient and the preferential binding parameter which are reviewed by Ruckenstein and Shulgin [15].



### 1.3 SDS in proteomics

Many proteins are poorly soluble in aqueous solution due to the presence of hydrophobic amino acids. Membrane proteins are a class of proteins that are associated with the cell lipid bilayer; these proteins tend to have more hydrophobic regions which can interact more favorably with the lipid membrane than the surrounding water molecules. Integral (transmembrane) proteins are permanently bound to the membrane, with highly hydrophobic segments of the protein often spanning the membrane itself. Peripheral membrane proteins are temporarily bound to the lipid membrane surface, or to integral membrane proteins. The importance of membrane proteins comes from their role in cell signaling, and regulation of cellular communication. It is estimated that out of all the proteins in the mammalian genome, 30% are classified as membrane proteins [16]. Membrane proteins represent two thirds of protein targets for potential drugs due to their accessibility [17]. However, these protein targets are often underrepresented in proteomics analysis, owing to reduced solubility.

To improve protein solubility, particularly for membrane proteins, sodium dodecyl sulfate (SDS) may be added [18]. SDS monomers bind to the protein *via* electrostatic and hydrophobic interactions. The hydrophobic binding of SDS to proteins is caused by the relative increase in entropy for interaction of the aliphatic chain of the SDS monomer with hydrophobic regions of the protein, relative to that of interactions between these same respective regions with water molecules. In other words, hydrophobic molecules prefer to come together in aqueous solution. Simultaneously, the electrostatic interactions of SDS with protein are readily recognized; electrostatic attraction between opposing charged ions are a direct result of Coulombic forces. The negatively charged head group of the SDS monomer attracts to cationic side chains of the protein (e.g., deprotonated glutamic and aspartic acid). At SDS concentrations above the critical micelle concentration (CMC), SDS micelles begin to form on the protein around the original monomer. The protein becomes denatured, causing loss of secondary, quaternary and tertiary structure due to replacement of previously favored intra-protein hydrophobic attraction to the more favored SDS-protein interaction [19]. The overall increase in protein solubility is related to the incorporation of the insoluble portions of the protein into the core of the soluble SDS micelle that forms upon these regions. Reynolds and Tanford showed that above 0.5 mM, SDS and protein bind together at a 1.4–1 mass ratio of SDS monomer to protein [20].

Surfactants such as SDS can also be used to disrupt cell membranes which will extract proteins from biologically relevant samples. Lipid membrane disruption and protein solubilization results in the removal of lipid monomers and effective solubilization of membrane proteins [18].

Further to the benefits of membrane extraction and protein solubilization, SDS also has important uses for enhancing protein solubility. Specifically, SDS is a key component of mass-based protein separation by polyacrylamide gel electrophoresis (SDS PAGE). More recently, the introduction of a related technology termed GELFrEE allows for intact protein separation with recovery of fractions sorted by size in the solution phase [21]. Unfortunately, with GELFrEE, samples are collected in the running buffer. Just as in SDS PAGE, the GELFrEE running buffer contains significant quantities of SDS. Therefore, for GELFrEE fractionated proteins to be amenable to LC-MS analysis, the samples must first be purified to remove the surfactant to levels that no longer interfere with ESI-MS sensitivity. A maximal level of 100 ppm SDS is often quoted to permit LC-MS analysis [22]. However, optimal MS sensitivity is only achieved when SDS is reduced to ~10 ppm or less [23]. For a sample initially containing 0.1% SDS, this would constitute >99% removal of SDS from the sample.

## 2. Protein purification strategies

A number of methods have been reported for the removal of SDS from proteomics samples, both at the peptide and at the intact protein level. These include classical dialysis [20], ultrafiltration [24], solid phase extraction [25], electrophoretic approaches [23], and protein precipitation [22]. These methods vary greatly in terms of their expected protein yield, level of purity, as well as their throughput and relative ease of use. The ideal SDS removal technique would provide *quantitative* recovery, with *completely* SDS removal from protein or peptide samples. While such a technique does not currently exist, some techniques have proven to be very close.

A promising approach to SDS removal was proposed by Wiśniewski et al., being described initially as a “universal” protocol for protein sample preparation [24]. The technique, known as filter aided sample preparation (FASP), relies on molecular weight cut off (MWCO) filters (~3 to 10 kDa in porosity) to selectively remove low molecular weight contaminants including SDS, while retaining the high mass proteins. Following purification by filtration, proteins are enzymatically digested on the filter to liberate peptides which are collected for MS analysis. While the technique is generally very effective at depleting SDS to levels permitting MS analysis, several researchers have noted the variable, if not poor recovery of proteins when processed by FASP, which may fall below 50% [26–29]. Protein loss can be attributed to the poor retention of low-mass proteins (<5 kDa), peptide fragments that do not readily pass through the filter (>5 kDa), or protein interactions with the filter giving rise to poor protein/peptide solubility. Nonetheless, FASP remains a favored approach to handle SDS-containing samples, owing perhaps to the ease of using disposable filter cartridges which generally provide positive proteome results. Perhaps the popularity of this technique also falls to a perceived lack of favorable alternative.

Consider dialysis as an alternative SDS depletion strategy; it should be recognized that this classic protein purification approach is, in fact, ineffective for the purpose of SDS depletion ahead of LC-MS analysis. With conventional dialysis, low molecular weight components may be eliminated by passive diffusion across a molecular weight cut-off membrane. Given an appropriate concentration gradient, and sufficient time one would assume the technique could eventually deplete SDS from a sample. However, the tight binding interaction between SDS and protein implies a significant portion of the SDS will remain complexed to the larger protein molecules. These bound surfactants will not be eliminated, even with exhaustive dialysis. For optimal MS analysis, both free and protein-bound SDS must be removed. Therefore, to fully deplete SDS from the sample, the interaction between SDS and protein must be overcome. FASP facilitates this depletion by adding compounds which weaken the SDS-protein interactions (e.g., urea, sodium deoxycholate) [30].

Similar to dialysis, several chromatographic approaches only offer partial depletion of SDS. Size exclusion chromatography for example, exploits the size difference between SDS and protein, but would not intrinsically separate SDS-protein complexes. Similarly, ion exchange chromatography can be used to selectively capture negatively charged SDS (strong anion exchange) [31], or positively charged proteins (strong cation exchange) [25], while allowing the opposing compound to flush through. While recognizing that multiple chromatographic approaches have been successfully incorporated into detergent-based workflows, it is noted that the existence of SDS-protein complexes implies that residual SDS is likely, and thus MS analysis is below optimal performance.

### 3. Protein precipitation

As with all purification strategies, maintain high protein recovery is of utmost importance. Biased sample loss can lead to incomplete proteome characterization, or may skew quantitative analysis by mass spectrometry. With this in mind, consider protein precipitation as a potential means for detergent purification ahead of LC-MS. Precipitation is recognized as a classic approach, predating chromatography and even electrophoresis. It has been used to isolate specific proteins, generally through the addition of a precipitating agent which acts to lower the solubility of the protein. Common precipitating agents include salts [32], organic solvents [22], acids [33], or polymers [34]. Heat and mechanical agitation may also lead to precipitation. In all cases, precipitating agents act to alter the interaction between protein and the solvent system (typically water). Often this occurs in parallel with modifying the tertiary structure of the protein. Once the proteins are made insoluble, they will aggregate and can be isolated from the supernatant solvent, which still contains the interfering contaminants.

Early scientific reports of protein precipitation focused on the isolation of specific proteins from sources including milk or plasma. The classic work by Hofmeister and his student Lewis in 1888 [35], measured the ability of a variety of salts to precipitate proteins from egg albumin and other sources. It was found that the precipitating ability of a salt was dependent on (1) the type of salt/precipitating agent used, (2) the type of protein, and (3) the concentration of protein. These factors seem to play a role in other forms of protein precipitation. Today, ammonium sulfate is still commonly used to precipitate and concentrate proteins from solution. An advantage of salt precipitation is its ability to selectively precipitate proteins from a solution, thus lending a form of separation. For example, Jiang et al. applied ammonium sulfate precipitation to deplete the highly abundant albumin protein from plasma [36]. In their report, 30%  $\text{NH}_4\text{SO}_4$  was used to precipitate proteins from plasma while leaving albumin in solution.

By contrast to salt, organic solvents can be used to induce precipitation of proteins. Precipitation has been seen through the use of a variety of water miscible solvents such as ethanol, acetonitrile, methanol, and acetone. Focusing on acetone precipitation, the solvent was first reported a century ago as a method to remove water from blood in an attempt to obtain a possible blood alternative [37]. Since then acetone has been applied in a number of applications including protein concentration, metabolite isolation and SDS detergent removal. Protein precipitation as a whole is a very common sample preparation strategy; in 1990 it was estimated that 80% of all proteomic experiments contained a protein precipitation step [38]. However, focusing on proteome analysis with MS, the popularity of protein precipitation did not keep pace with alternative strategies including chromatographic approaches. This is most likely due to two factors: (1) the *apparent* variable protein recovery, as reported across different labs and for different proteins, (2) a *perceived* difficulty in isolating the protein pellet through manual pipetting. As is described below, both of these shortcomings of solvent precipitation have now been overcome.

#### 3.1 Acetone precipitation mechanism

Protein solubility is a physiochemical property which describes the amount of protein that can be solvated and therefore dissolved by a given solvent. As has been already described, sample additives such as SDS can influence protein solubility, as can protein specific factors include the amino acid sequence, molecular weight, and protein conformation. Altering the solvent conditions will have an influence on



protein solubility, noting particularly the solution pH, ionic strength, temperature, and solvent polarity. The addition of organic solvent to a water soluble protein generally lends a reduction in protein solubility, leading to protein aggregation. This ultimately is caused by increasing the protein-protein interactions (electrostatic or hydrophobic attractions) while decreasing the solvation ability of the solvent.

As a starting point, consider that it is possible to induce selective protein precipitation by using a modestly low concentration of organic solvent (if not a purely aqueous system) and controlling the pH of the solution. Proteins are known to have reduced solubility at their intrinsic isoelectric point (pI) due to the decrease of electrostatic charge on the surface of the protein; this decrease charge results in a decrease in interaction between water and the macromolecule and protein-protein interactions dominated. Although proteins have a reduced solubility at their pI, they are not necessarily insoluble. Through the addition of low concentrations of organic solvent the reduced solubility can be further reduced causing precipitation. This allows for the selective removal of proteins according to isoelectric point. This is the basis of a technique known as Cohn fractionation of blood plasma, where proteins such as human serum albumin, serum gamma globulin, fibrinogen, thrombin, and a few others are isolated from plasma [39].

Precipitation induced through the addition of organic solvent is thought to be caused by the decrease in the dielectric constant of the solution (water has a dielectric ~70 while most organic solvents are ~20). As the amount of organic solvent is increased, the solvating power of water will decrease. It has been shown that ethanol binds water more strongly than it does proteins [40]. This is thought to extend to other organic solvents. The decrease in solvation causes the hydration sphere around the proteins to shrink. The overall shielding of charged region is therefore decreased. Following this, it is thought that the increased electrostatic interaction between *opposing* charged regions on distinct protein molecules will cause the proteins to aggregate. As described below:

$$|F| = k_e \frac{|q_1 q_2|}{r^2} \quad (4)$$

$$k_e = 1/(4\pi\epsilon_0\epsilon) \quad (5)$$

F is the magnitude of the force between the charges (for like charges, this is a repulsive force; opposing charged species attract),  $q_i$  is the magnitude of charge,  $r$  is the distance between the two charges,  $\epsilon_0$  is the permittivity of free space, and  $\epsilon$  is the relative permittivity of the solution. As can be seen, the dielectric constant is in the denominator of the equation, meaning that the magnitude of the Coulombic force increases as the dielectric decreases [41, 14]. Water has a high dielectric, while organic solvents have lower dielectrics. Therefore, charged species are more strongly attracted in organic solvent. Also, it is noted that electrostatic interactions are not the only force to consider. Dipolar van der Waals forces are thought to play an important role as well in bringing proteins together. However, as presented below, there are issues with the underlying assumption of this model, which bring to question the validity of this model.

The addition of acetone to protein samples is generally performed at low temperature. This is done to prevent protein denaturation which has been found to occur quickly above 10°C [41]. The denaturation effect of organic solvent is due to the interaction of the more favorable interaction of the hydrophobic regions of the protein compared to water. This decreases the entropic loss which occurs when the protein unfolds, promoting denaturation. The use of cold temperatures is thought to reduce the conformational flexibility of proteins preventing the organic solvent



from accessing the hidden hydrophobic regions. Acetone has been found to be less denaturing than ethanol for protein precipitation.

### 3.2 The role of salt in acetone precipitation

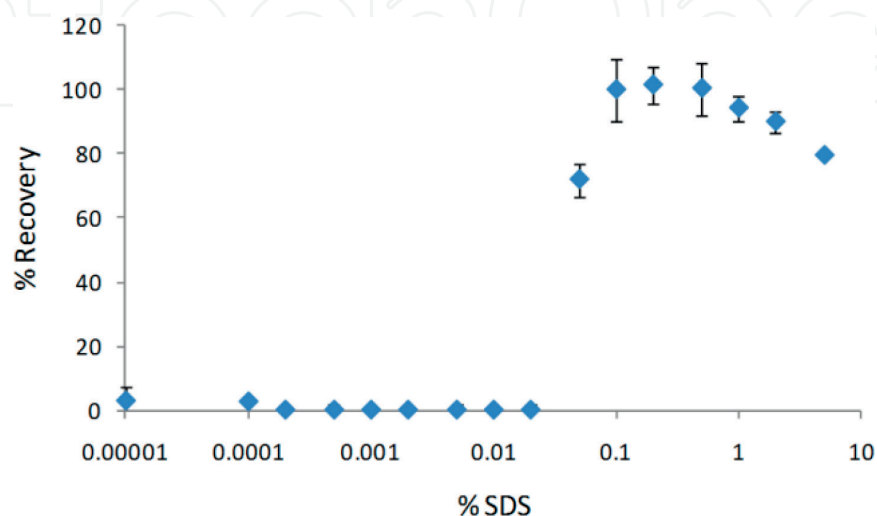
The current theory of solvent precipitation described above is limited in its ability to explain a more recent finding related to organic solvent precipitation. Specifically, experimental evidence has brought to question the validity of electrostatic attraction *between proteins* as a fundamental premise of protein precipitation in organic solvent.

Work in our lab has shown that for a series of protein standards, as well as for complex protein mixtures, the addition of 80% acetone does not in itself induce the precipitation of proteins [42]. In other words, it has been shown that all proteins are in fact completely soluble in 80% acetone! Such a finding is certainly surprising, as 80% acetone. However, upon addition of even minimal amounts of salt (sodium chloride, or other ionic species), these proteins will immediately precipitate from the organic solvent system [42]. **Figure 1** depicts this trend for the protein bovine serum albumin (BSA) precipitated in the presence of increasing concentrations of SDS.

While it may be easy to assume that the phenomenon is related to the solubilizing or denaturing properties of SDS, this assumption does not explain why the absence of SDS prevents the protein from precipitating. In fact, it was discovered that it was the ionic character of SDS that gave rise to this trend. Identical plots can be produced using NaCl in the precipitating solvent system; only above a threshold level will the protein begin to precipitate.

In this study, it was shown that the amount of salt required to induce quantitative protein precipitation was dependent on (1) the concentration of acetone in the solvent system, (2) the type of protein in the sample, (3) the concentration of protein in the sample [42].

To explain why traces of salt are needed to induce near-quantitative precipitation of proteins in acetone, we propose a theory of ion pairing in organic solvent. This theory is only a slight perturbation of the prevailing theory that protein aggregation is related to electrostatic attraction. The primary difference simply relates to the identity of the species being attracted.



**Figure 1.**

The recovery of bovine serum albumin (initially 1 g/L) following precipitation in 80% acetone with inclusion of increasing concentrations of SDS in the initial solution. When low concentrations of SDS are present, poor recovery is obtained, implying that the protein is still soluble in 80% acetone.

In aqueous systems, ionic species exist in solution as hydrated spheres, the water acting to partially shield the charge of the ion. This shielding prevents opposing charged ions to attract (pair) as they otherwise would. This is essentially the premise of Coulomb's law. Likewise, it is understood that proteins also carry surface charge (both positive and negative charges can exist simultaneously). As has already been described when considering protein solubility at its isoelectric point, the primary reason for protein solubility in water relates to electrostatic repulsions. This is of course why proteins are least soluble at a pH equal to their respective isoelectric point.

Suppose now that a protein is placed in a solvent system of lower dielectric strength, such as acetone. The hydration spheres surrounding the charges will be reduced, allowing the exposed ions of opposing charge to experience a higher attractive force—according to Coulomb's law. What is essential to realize is that it is the attraction between protein and salt that are of interest during solvent precipitation. Whereas in water, the salt ions are hydrated, in organic solvent the lower dielectric allows these ions to pair with opposing charged residues on the proteins [43]. The net result is to effectively neutralize the surface charge of the protein. Adding salt in organic solvent essentially performs the same task as titrating the solution pH to the protein's isoelectric point. Once the protein's surface charge is neutralized, the repulsive electrostatic forces between proteins are minimized. This allows van der Waals forces to take over, allowing the hydrophobic portions of proteins to aggregate. Precipitation is therefore intrinsically connected to electrostatic effects. However, these specific interactions are also tied to the presence of ionic species (salts) in the system.

### **3.3 Protein recovery and purity from acetone precipitation**

Throughout the literature, variable protein recovery has been reported through acetone precipitation. Certainly, in light of the more recent findings that salt is required for protein recovery in acetone, low yield may potentially be explained by a lack of understanding of the variables influencing protein recovery. It has been suggested that recovery through acetone precipitation is protein specific, and also depends on sample concentration, as well as the presence of sample additives (beyond salt). Reported recoveries from acetone precipitation have ranged from extremely low to near quantitative.

Thongboonkerd et al. measured the protein recovery from urine using acetone precipitation [44]. Increasing the percentage of acetone (from 10 to 90%) improved protein recovery, though only 40% yield was obtained in 90% acetone. Barritault et al. found acetone precipitation provided high (>95%) in a short period of time for high concentrations of samples but required a far greater time (overnight) to provide similar yield to more dilute samples [45].

Srivastava and Srivastava employed 50% acetone to enrich gamma-crystallin from human eye lenses [46]. The hydrophobic, low molecular weight (20 kDa) protein remains soluble in a 50% acetone solution. Ashri et al. examined the effect of acetone concentration on removing proteins from plasma and report that only 70% acetone is required to induce near quantitative precipitation of proteins [47]. As concentration was decreased, yield too decreased. Lin et al. determined that an 85% acetone solution produced optimal precipitation, however it is noted that 80% was only marginally lower in efficiency [48].

Low recovery ranging from 40 to 50% was obtained by Sickmann et al. when purifying human cerebrospinal fluid [29]. However, Yuan et al. found recoveries of 94% when applying acetone to a similar sample of human cerebrospinal fluid [49]. No significant difference in protocol was reported, implying that recovery

differences are either related to an unreported difference, or are perhaps related to accidental sample loss contributed through manual sample pipetting.

Using the newly established principle of including sufficient quantities of salt (10–100 mM) to ensure maximal protein recovery, our group has consistently reported protein recovery above 90%. Often times, recovery is statistically considered to be quantitative (>99%). Under appropriate conditions, high protein recovery is obtained for all sample types (soluble/hydrophobic, high/low molecular weight) and over a range of protein concentrations (sub microgram to high milligram per milliliter starting amounts). Furthermore, the ability of acetone precipitation to eliminate SDS has been examined by Botelho et al. [22]. In this report, a sample initially containing 2% SDS can be depleted below 0.005% with inclusion of an additional washing step to rinse the protein pellet. Still, to obtain high recovery and purity, the pipetting step must be performed with great precision.

### 3.4 Issues with protein precipitation

While a so-called “universal” strategy for front-end sample preparation does not exist, it can be suggested that acetone precipitation presents a powerful starting point. Despite the potential for high yield and purity, a number of potential disadvantages exist. Here we focus attention on the potential of acetone to induce chemical modifications in the sample, as well as difficulties in pipetting the sample.

#### 3.4.1 Protein modification by acetone

Simpson et al. described a +40 Da peptide modification induced by the presence of acetone in solution [50]. They correlate this modification to peptide sequences which contain a glycine residue as the second amino acid in the sequence. They found modifications to exist within 1 hour of reaction and a rate constant of  $0.29 \pm 0.01 \text{ h}^{-1}$  for a number of different peptides. There was no evidence to suggest that acetone can modify at the protein level. However this is still an issue in bottom-up proteomics as any unknown peptide modification would skew results through the splitting of signal intensity leading to incorrect conclusions. The complete removal of acetone after precipitation is required to prevent this modification, therefore proper steps should be taken to guarantee complete removal such as allowing significant time for the pellets to air dry, or drying under vacuum.

In an independent study, it was shown that acetone may induce modifications at the intact protein level, manifesting as a +98 u mass shift in the mass spectrum of acetone-precipitated proteins [51]. The +98 u artifact was speculated to originate from the aldol condensation of acetone to form diacetone alcohol and mesityl oxide, which in turn reacts by nucleophilic attack. The degree of protein modification was temperature and time sensitive, wherein over 90% of cytochrome c was detected in the modified form following 1 hour incubation in acetone at 0°C. However, it was also shown that this modification was highly dependent on solution pH. By precipitated the protein in an acidic environment, any possibility of modifying the protein was eliminated.

#### 3.4.2 Technical difficulties of protein precipitation

The second disadvantage of acetone precipitation over competing sample preparation techniques such as filtration or chromatography involves the technical difficulty in separating the protein pellet from the organic solvent. If one removes too little solvent, contaminants will remain in the protein sample which may interfere with subsequent analysis. However if too much supernatant is removed,

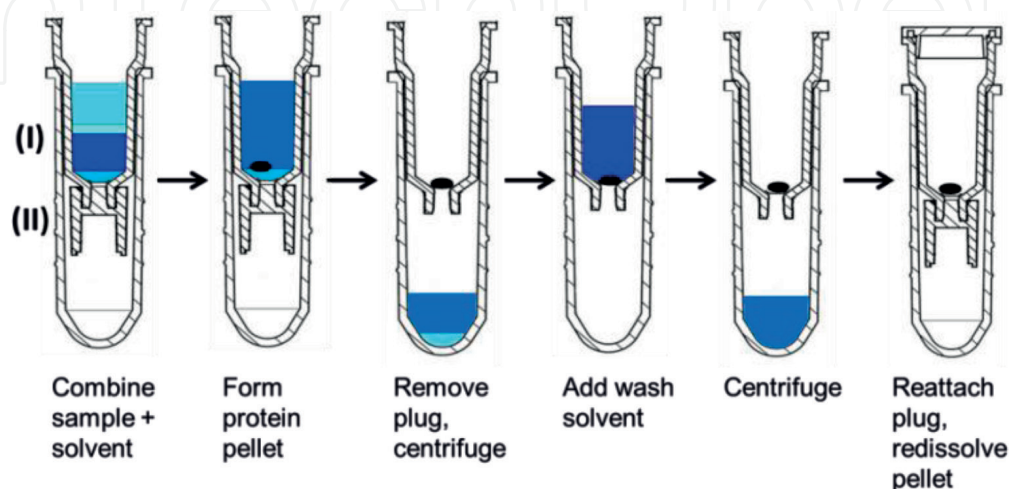
disruption and loss of the protein pellet can occur which will cause reduce protein yield. This is especially the case when precipitating dilute protein samples; here the protein pellet may not even be visible to the naked eye. Protein losses due to accidental pipetting of the pellet are almost unavoidable, without considerable care, and a strong familiarity of the technique. Botelho et al. suggest the use of a wash step to allow one to leave behind larger portions of acetone; however each wash implies another manipulation which takes time, and introduces the possibility of error [22]. Improper formation of the protein pellet after the wash may also cause protein loss. The pellet is not necessarily strongly bound to itself, or to the vial surface. Regardless of the inherent ability of acetone precipitation to aggregate protein, the method is not useful without a simple means of isolating the pellet.

### 3.5 The ProTrap XG

Filtration cartridges are commonly used in multiple applications such as DNA isolation, trapping cells, and of course for protein isolation. A number of micro-centrifuge filtration devices are currently available on the market. However, these devices are not inherently designed to recover precipitated precipitation. With precipitation, a certain period of time is required to allow the protein to aggregate. Thus, filtration should not be initiated until after the pellet has properly formed. However, once the protein has aggregated, the macroscopic structure should enable filtration as a reliable means of isolating the pellet. Following precipitation, the protein pellet must now be resolubilized, which again requires the absence of filtration during this stage.

The ProTrap XG is a disposable spin cartridge used to recover precipitated protein. As shown in **Figure 2**, the cartridge includes a detachable plug at the base of a Teflon membrane filter. Compared to molecular weight cutoff cartridges (seen in dialysis or FASP), the ProTrap XG membrane is of relatively large porosity, allowing rapid flow of solvent through the cartridge. However, the membrane is still sufficient to recover aggregated protein in high yield. As demonstrated, the ProTrap XG allows near quantitative recovery of proteins (**Figure 3**), including sub microgram levels [52].

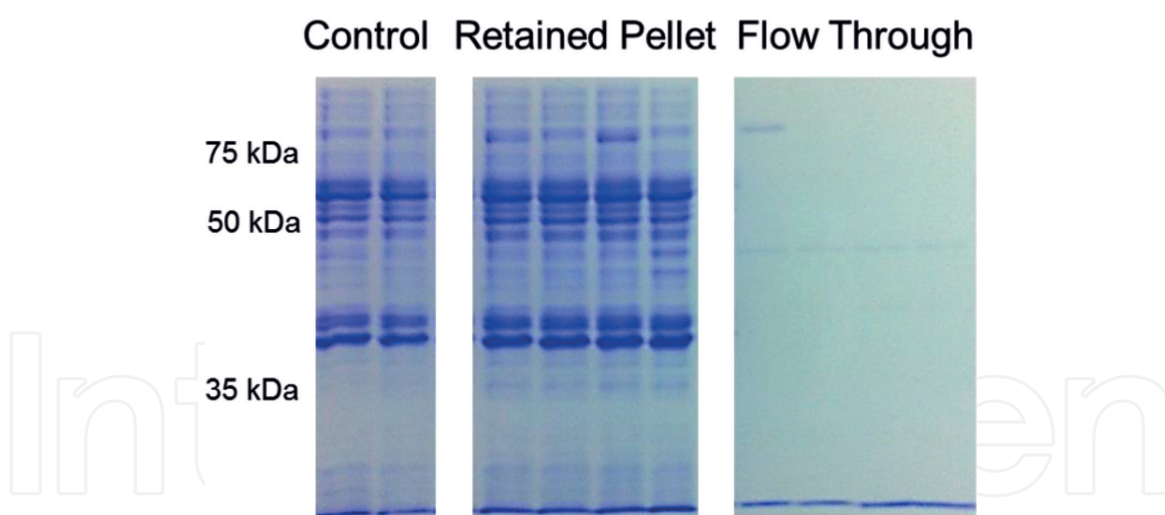
The ProTrap XG also includes a solid phase extraction cartridge which can be attached to the base of the filter. Such cartridge enables downstream cleanup or separation of proteins or peptides following the precipitation step.



**Figure 2.**

*The ProTrap XG is a two-stage cartridge combining an upper membrane filtration cartridge (I) with a detachable plug (II) to capture precipitated proteins. Acetone precipitation of proteins with the ProTrap XG allows for recovery in high yield and with high purity.*





**Figure 3.**

*Acetone precipitation of a proteome mixture using the ProTrap XG demonstrated high recovery of the aggregated protein above the membrane filter.*

### 3.6 How to resolubilize proteins after precipitation

Perhaps the main disadvantage of protein precipitation is that the resulting solid protein pellet is not directly amenable to MS analysis. Specifically, the protein must first be resolubilized prior to further analysis. Although a number of strategies exist, currently there are no “perfect methods” to allow for resolubilization of the protein pellet. Any solubilization method must be compatible with downstream analysis. Naturally, this means that SDS is not available as a solubilizing additive. The simplest approach is to simply use water, or more specifically the solvent system used for LC-MS analysis (typically 5% acetonitrile, 0.1% formic acid in water). The addition of 5% acetonitrile does little to aid protein dissolution, though pH is an important addition.

A number of MS-compatible “cleavable” surfactants have been proposed to resolubilize proteins [53]. But they are generally quite expensive. High concentrations of urea (8 M) are common to resolubilize proteins. This additive is readily removed through reversed phase, so it can be considered compatible with LC-MS. Also, the addition of digestive enzymes including trypsin will aid in the dissolution of proteins, by liberating the more soluble peptide counterparts into solution. As a disadvantage, such an approach is only suited to bottom-up proteomics.

An effective strategy to dissolve intact proteins is to use high concentrations of the organic acid, including formic acid (50–80% acid by volume). This solvent system is shown to dissolve proteins as effectively as high concentrations of SDS [54]. A concern with formic acid is its propensity to rapidly modify the protein, causing the addition of +28 u adducts in the resulting mass spectrum (28 = CO modification). However, this reaction can be reduced, if not entirely eliminated by maintaining a low temperature during incubation of the protein in concentrated formic acid [55]. At  $-20^{\circ}\text{C}$ , no protein modifications are detected. At the same time, the reduced temperature does not deter proteome resolubilization. Finally, formic acid is compatible with LC-MS analysis, as the proteins are retained on the reversed phase column while the acid flushes through.

## 4. Conclusions

Proteome analysis by LC-MS demands isolation of proteins in high purity, particularly when the sample is contaminated with known MS interferences such

as SDS. While several options are available for protein purification, only some are suited to remove the SDS that is tightly associated with protein. Solvent precipitation with high concentrations of acetone shows promise as a high recovery, high purity approach for proteome sample preparation. The requirement to include ionic species (salt) in the precipitating solvent lends new knowledge to the underlying mechanism controlling solvent precipitation. So long as the supernatant can be removed without disturbing the protein pellet, exceptional high recovery and purity can be expected. This is facilitated by filtering the sample using disposable spin cartridges. The resulting pellet can be resolubilized in MS compatible solvents such as concentrated formic acid. This workflow presents an effective approach to detergent-based proteome analysis in both top-down and bottom-up formats. As such, acetone precipitation should see increased use as researchers exploit the advantages of SDS for protein extraction and separation.

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## Conflict of interest

A.D. holds a patent on the ProTrap XG, and is affiliated with Proteoform Scientific, the company who holds license to commercialize and distribute the ProTrap XG. All results presented in this chapter have been verified through independent peer review and the original scientific articles have been cited accordingly.

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